

Regulation of Human Chondrocyte Function through Direct Inhibition of Cartilage Master Regulator SOX9 by MicroRNA-145 (miRNA-145)*

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Background: SOX9 is essential for cartilage.

Results: miR-145 directly targets SOX9, and increased miR-145 levels reduce expression of SOX9 and the extracellular matrix genes critical to cartilage function.

Conclusion: SOX9 is subject to significant post-transcriptional regulation by miR-145 in human chondrocytes.

Significance: Our data give new insights into the mechanisms regulating SOX9 and identify miR-145 as a new target for cartilage repair.

Articular cartilage enables weight bearing and near friction-free movement in the joints. Critical to its function is the production of a specialized, mechanocompetent extracellular matrix controlled by master regulator transcription factor SOX9. Mutations in SOX9 cause campomelic dysplasia, a haploinsufficiency disorder resulting in severe skeletal defects and dwarfism. Although much is understood about how SOX9 regulates cartilage matrix synthesis and hence joint function, how this master regulator is itself regulated remains largely unknown. Here we identify a specific microRNA, miR-145, as a direct regulator of SOX9 in normal healthy human articular chondrocytes. We show that miR-145 directly represses SOX9 expression in human cells through a unique binding site in its 3'-UTR not conserved in mice. Modulation of miR-145 induced profound changes in the human chondrocyte phenotype. Specifically, increased miR-145 levels cause greatly reduced expression of critical cartilage extracellular matrix genes (COL2A1 and aggrecan) and tissue-specific microRNAs (miR-675 and miR-140) and increased levels of the hypertrophic markers RUNX2 and MMP13, characteristic of changes occurring in osteoarthritis. We propose miR-145 as an important regulator of human chondrocyte function and a new target for cartilage repair.

Cartilage master regulator transcription factor SOX9 is essential for cartilage development in mice (1), whereas heterozygous mutations in the human SOX9 gene cause the severe skeletal malformation syndrome campomelic dysplasia (2). Decreased SOX9 expression underlies the loss of the differentiated phenotype of human articular chondrocytes during subculture *in vitro* and represents the major shortcoming of current cell-based cartilage repair therapies (3, 4). In addition, SOX9 levels are suppressed in human osteoarthritic cartilage (5), and this most likely contributes to the phenotypic instabil-

ity observed in osteoarthritis. Transcription factor SOX9 has been shown to be essential for expression of the differentiated chondrocyte phenotype by directly activating genes coding for key cartilage extracellular matrix components such as Col2a1 (6) and aggrecan (7). However, less is known about the mechanisms controlling expression of SOX9 itself, although we have previously shown that SOX9 is regulated by hypoxia via transcription factor HIF-2 α ² in human articular chondrocytes (4).

MicroRNAs (miRNAs) are small non-coding RNA molecules of 21–23 nucleotides that control gene expression at the post-transcriptional level and have been shown to play a vital role in a wide variety of biological processes, whereas dysregulated expression of miRNAs is found in many pathological conditions (8) including osteoarthritis (9). The most studied miRNA in cartilage is miR-140, the level of which is relatively high in normal articular cartilage but reduced in osteoarthritic tissue (10). miR-140 knock-out mice are viable but develop osteoarthritic-like changes with age (11). Recent work in our laboratory has uncovered another microRNA, miR-675, which was shown to control expression of key cartilage matrix gene COL2A1 in human articular chondrocytes (12). Interestingly, expression of the primary miRNA transcripts for both miR-140 and miR-675 is regulated by SOX9 (12, 13), thus further extending the known influence of this master regulator on cartilage function.

Here we show that miR-145, an miRNA whose main role to date has been linked to vascular smooth muscle cell maintenance (14, 15) and human embryonic stem cell differentiation (16), can directly target SOX9 in normal human articular chondrocytes through binding a specific site in its 3'-UTR, which interestingly is not conserved in mice. Levels of miR-145 inversely correlate with protein and mRNA levels of SOX9 during human articular chondrocyte dedifferentiation. miR-145 overexpression in articular chondrocytes obtained from non-arthritic patients greatly reduced the levels of SOX9, together with the

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² The abbreviations used are: HIF-2 α , hypoxia-inducible factor 2 α ; miRNA, microRNA; miR-145, microRNA-145; RT-qPCR, RT-quantitative PCR; D1, Deletion 1; D2, Deletion 2; ADAMTS5, a disintegrin-like and metalloprotease with thrombospondin motifs 5.

critical cartilage matrix components COL2A1 and aggrecan, whereas undesirable hypertrophy markers were significantly increased. Thus, we have identified miR-145 as a direct regulator of master transcription factor SOX9 with critical effects on human articular chondrocyte function, and we propose miR-145 as a promising target for cartilage repair.

EXPERIMENTAL PROCEDURES

Harvest of Human Articular Cartilage and Chondrocyte Culture—Healthy articular cartilage was obtained from patients after informed consent and following local ethics committee guidelines. Cartilage was harvested from the femoral condyle and tibial plateau following amputation due to sarcomas not involving the joint space. In total, tissue was obtained from 13 donors (average age of 38 years) (for full details, see Table 1). Cartilage specimens were collected on the day of surgery and cut into small pieces (1–2 mm³). Diced cartilage was placed in 1.5 mg/ml collagenase type 2 (Worthington®) with DMEM containing 10% fetal calf serum (FCS) (Biosera) and incubated at 37 °C for 18 h with shaking. Isolated human articular chondrocytes were then passed through a cell strainer, pelleted, and washed twice with medium. Cells were seeded at a density of 8×10^3 cells/cm² in DMEM with 10% FCS. Passage 0 cells were subcultured after 5–7 days of isolation and either used directly (P1 cells) or passaged once more (P2 cells) before experiments.

RNA Extraction, Reverse Transcription, and Real Time PCR—Total RNA including small RNA was extracted from human articular chondrocytes using TRIzol (Invitrogen) according to the manufacturer's instructions. For microRNA detection, 500–1000 ng of RNA were reverse transcribed using the TaqMan microRNA reverse transcription kit (Applied Biosystems), including the relevant microRNA-specific primer (Applied Biosystems) in the reaction. Up to three specific primers were used simultaneously per reverse transcription reaction. Reverse transcription was followed by real time PCR with a TaqMan PCR master mix (Applied Biosystems) and the appropriate microRNA-specific TaqMan probe (Applied Biosystems). For mRNA detection, 200–500 ng of RNA were reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems) including random primers. Reverse transcription was followed by real time PCR with a SYBR Green PCR master mix (Applied Biosystems). The specific primers used for the different mRNAs were: AGGRECAN, 5'-CGTGC-GCCCATCAACA-3' and 5'-ACACCTCCCCCTCAAGTC-TGT-3'; COL2A1, 5'-GGAAGAGTGGAGACTACTGGATT-GAC-3' and 5'-TCCATGTTGCAGAAAACCTTCA-3'; H19, 5'-ACCCACAACATGAAAGAAATGGT-3' and 5'-GAGGG-TTTTGTGTCCGGATT-3'; SOX9, 5'-CGCCATCTTCAA-GGCGTGC-3' and 5'-CCTGGGATTGCCCGAGTGC-3'; MMP13, 5'-TTACCAGACTTCACGATGGCATT-3' and 5'-TCGCCATGCTCCTTAATTCC-3'; RUNX2, 5'-CAGGCAG-GTGCTTCAGAACTG-3' and 5'-GCATTCGTGGGTGGA-GAA-3'; ADAMTS5, 5'-GCTCACGAAATCGGACATTT-ACT-3' and 5'-GGAACCAAAGGTCTCTTCACAGAAT-3'; RPLP0, 5'-CCATTGAAATCCTGAGTGATGTG-3' and 5'-CTTCGCTGGCTCCCACTTT-3'; 28S, 5'-TTGAAAT-

CCGGGGGAGAG-3' and 5'-ACATTGTTCCAACATGC-CAG-3'.

Transient Transfections and Luciferase Assays—First or second passage human chondrocytes were seeded at 2.4×10^5 cells per 3.5-cm dish and transfected 1 day after seeding. Transfection with control or miR-145 inhibitors or precursors (Ambion) was carried out at a final concentration of 100 or 5 nM, respectively, using Lipofectamine 2000 (Invitrogen) for 4 h in Opti-MEM I. Following transfection, medium was changed with pre-equilibrated DMEM (in 20 or 1% oxygen) containing 10% fetal calf serum. Cells were incubated in each oxygen tension for a further 24–40 h before termination of the experiment.

Plasmid DNA was similarly transfected with Lipofectamine 2000 into human articular chondrocytes (8×10^4 cells/well in a 12-well plate). After 24 h, cells were lysed, and luciferase activity was determined with the Dual-Glo luciferase assay system (Promega).

Plasmids—To construct the firefly luciferase reporter plasmids, including deletions of the predicted miR-145 binding sites, a primer pair was designed flanking nucleotides 273–295 (5'-ATTTCGCTGCTCCATTTA-3' and 5'-AAACCCTCTT-CAGAGCAA-3') of the human SOX9 3'-UTR (Deletion 1, D1) and a second primer pair flanking nucleotides 1381–1406 (5'-AAGAAAAAAGGGATGGAC-3' and 5'-GGAAACTTGT-TCTTTTTTTG-3') (Deletion 2, D2). The pSGG_Luc SOX9 3'-UTR plasmid (SwitchGear Genomics) was amplified by PCR reaction with each of the primer pairs and recircularized using a Phusion® site-directed mutagenesis kit (New England Biolabs). These newly designed constructs (pSGG_Luc SOX9 3'-UTR D1 and pSGG_Luc Sox9 3'-UTR D2, respectively) were sequenced to verify the deletion. pSGG_Luc SOX9 3'-UTR D1 includes deletion of the nucleotides 272–293 of SOX9 3'-UTR, whereas pSGG_Luc SOX9 3'-UTR D2 includes deletion of the nucleotides 1349–1427. To generate the plasmid containing both deletions simultaneously (pSGG_Luc SOX9 3'-UTR D1+D2), a region containing the nucleotides 272–293 in pSGG_Luc SOX9 3'-UTR D2 was substituted by the same sequence including deletion of the nucleotides 272–293 excised from pSGG_Luc SOX9 3'-UTR with XbaI restriction enzyme. This construct was also sequenced to verify the deletions.

Western Blotting—Human articular chondrocytes were cultured as monolayers in 20 or 1% oxygen tension for 36–48 h before lysis in radio-immunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid). SOX9 protein was detected by Western blotting using a polyclonal anti-SOX9 antibody (AB5809 Millipore) and ECL reagent (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.

Ribonucleoprotein Precipitation and miRNA Isolation—Immunoprecipitation of ribonucleoprotein was performed as described previously (23) in human articular chondrocytes with transfection of control or miR-145 precursors. Briefly, 5 million cells were lysed in 100 ml of ice cold polysome lysis buffer (5 mM MgCl₂, 100 mM KCl, 10 mM Hepes, pH 7.0, and 0.5% Nonidet P-40) with freshly added 1 mM DTT, 100 units/ml RNase OUT (Invitrogen), and Complete mini EDTA-free protease inhibitor mixture (Roche Applied Science, Basel, Switzerland) for 5 min.

miR-145 Directly Targets SOX9 in Human Chondrocytes

Centrifugation was carried out at $14,000 \times g$ at 4°C for 10 min. Supernatant was mixed with 900 μl of ice-cold NT2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl_2 , 0.05% Nonidet P-40) containing freshly added 200 units/ml RNaseOUT (Invitrogen), 0.5% vanadyl ribonucleoside (Invitrogen), 1 mM DTT, 15 mM EDTA, and 50 μl of mouse anti-human Ago2 (clone 2E12-1C9, Abnova, Taipei City, Taiwan)-coated Sepharose G beads (Abcam, Cambridge, UK). Incubation was carried out overnight at 4°C on a rocking platform. On the following day, beads were washed five times with ice-cold NT2 buffer and separated into two portions: one for RNA isolation to identify miRNAs and another portion for Western blotting to check for successful immunoprecipitation of Ago2. Mouse IgG1 isotype control (Abcam) was used as a negative control for the immunoprecipitation procedure. RNA (including miRNAs) from the IgG or Ago2 immunoprecipitations was isolated using TRIzol and glycogen (Invitrogen) as a carrier in the ethanol precipitation step. miR-145 detection was performed using a TaqMan specific assay as described above.

Statistics—Statistical significance was evaluated by two-tailed Student's *t* test. All data are shown as mean \pm S.E. *p* < 0.05 was considered statistically significant.

RESULTS

Levels of miR-145 Inversely Correlate with SOX9 Expression during Human Articular Chondrocyte Dedifferentiation—A TaqMan low density array was performed on RNA extracted from human articular chondrocytes from three different patients (showing no signs of joint disease). MicroRNAs potentially relevant to the differentiated chondrocyte phenotype were identified as those whose expression was altered with dedifferentiation through serial passage (data not shown). From this preliminary list, miR-145 was selected for validation using an individual TaqMan assay for the mature miRNA form because SOX9 mRNA was predicted to be targeted by this miRNA by the TargetScan software. miR-145 levels increased with loss of the differentiated chondrocyte phenotype, whereas SOX9 expression was decreased at the RNA and, more notably, at the protein level (Fig. 1). These experimental data, together with bioinformatic analysis, suggested that miR-145 could be directly involved in the repression of SOX9 levels during dedifferentiation. In humans (and larger animals), the avascular cartilage is hypoxic, and we have previously shown that hypoxia promotes the differentiated articular chondrocyte phenotype through HIF-2 α -mediated induction of SOX9 (Lafont *et al.* (4). Human chondrocytes showed increased levels of SOX9 upon exposure to hypoxia, whereas miR-145 expression remained unchanged (Fig. 1). Nevertheless, given that hypoxia is a normal physiological condition of chondrocytes in human articular cartilage, we performed subsequent experiments in hypoxia (in addition to normoxia, 20% oxygen).

miR-145 Negatively Regulates Endogenous SOX9 Expression in Human Articular Chondrocytes—To assess whether miR-145 was regulating SOX9, the mature 23-nucleotide miRNA was inhibited (with antisense chemically modified RNA-based inhibitors) or overexpressed in transient transfection experiments in human articular chondrocytes obtained from a range of donors following amputations due to sarcomas not involving

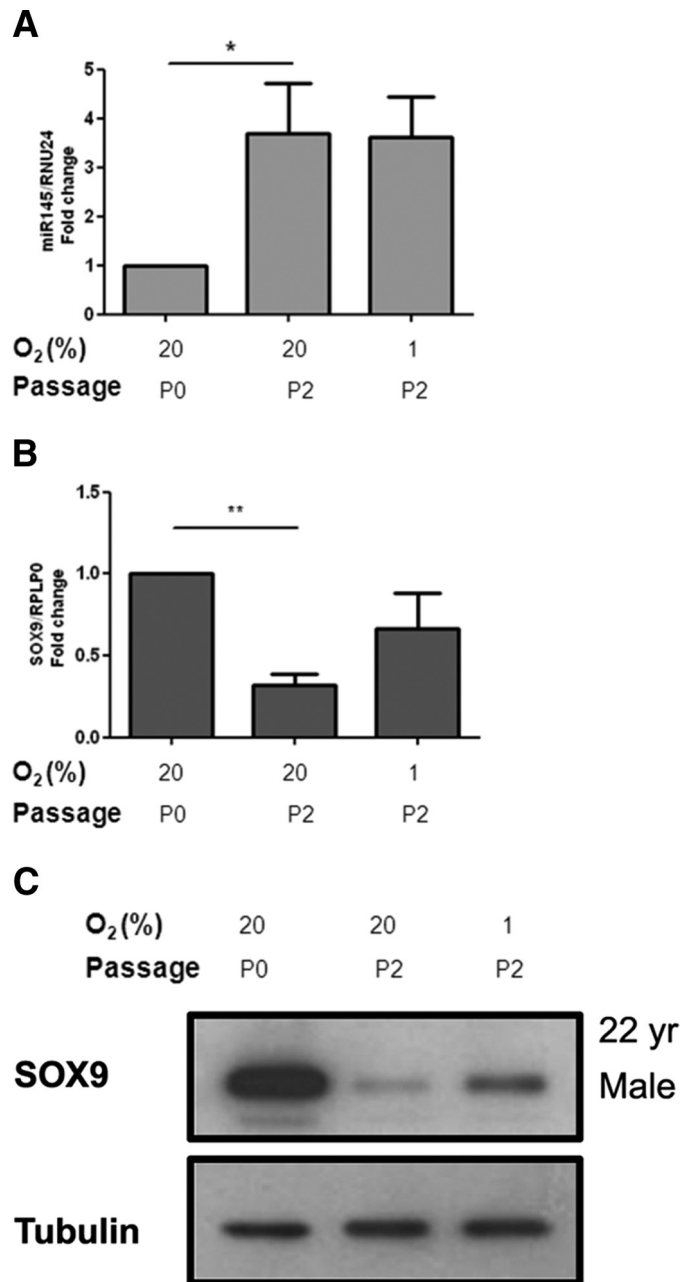


FIGURE 1. Levels of miR-145 inversely correlate with SOX9 during loss of differentiated phenotype in human articular chondrocytes. P0 denotes freshly isolated human chondrocytes kept in monolayer culture for 7 days in 20% O₂. P2 denotes chondrocytes that were dedifferentiated by two rounds of serial passage (approximately 3 weeks). P2 cells were then exposed to hypoxia (1% O₂) for 36 h to induce partial redifferentiation of the cells. A, miR-145 levels were assessed by RT-qPCR from human articular chondrocytes obtained from three different donors (*n* = 3). Average (\pm S.E.) values are shown, expressed relative to P0 levels (set to 1). B, SOX9 mRNA levels determined by RT-qPCR from the same cultures. C, Western blot showing SOX9 expression at the protein level in cells from a representative donor. Note: *, *p* < 0.05, **, *p* < 0.01.

the joint (Table 1). Transfection efficiency was high for pre-miRNA precursors but much lower for the miRNA inhibitors, with no negative effect on viability in either case (data not shown). The miRNA precursors used to overexpress the mature miRNAs are small, partially double-stranded RNAs that mimic endogenous precursor miRNAs. Mature miRNAs function as a part of the RNA-induced silencing complex, which is

TABLE 1**Donor details**

Human articular chondrocytes were isolated from a total of 13 different donors, with an average age of 38 years. Non-diseased cartilage was harvested from the knee following amputations due to soft tissue and osteosarcomas not involving the joint space.

Donor	Age (in years)	Sex
1	53	Female
2	65	Male
3	16	Male
4	23	Male
5	62	Female
6	53	Male
7	35	Male
8	23	Male
9	11	Male
10	16	Male
11	66	Female
12	47	Male
13	22	Male

the effector complex where miRNAs regulate the translation/stability of their target mRNAs. Association of the mature overexpressed miRNA with the main RNA-induced silencing complex component Argonaute2 was found to be 6-fold higher than endogenous levels and thus represents an unphysiological level of overexpression (Fig. 2A). Such miR-145 overexpression greatly decreased the levels of SOX9 both at the mRNA and particularly at the protein level (Fig. 2, B and C). Such results are consistent with a common mechanism of miRNA-mediated regulation where translational repression mediated by the miRNA is accompanied by partial degradation of the target mRNA (17). Interestingly, overexpression of miR-145 appeared to have a greater impact in hypoxic conditions and largely prevented the increase in SOX9 expression induced by hypoxia. miR-145 inhibition resulted in increased SOX9 protein levels (Fig. 2D), without significant changes in SOX9 mRNA levels (data not shown). We attribute the subtlety of the observed effect to the low transfection efficiency obtained for these molecules.

miR-145 Directly Targets SOX9 in Human Articular Chondrocytes Specifically through Nucleotides 266–288 in 3'-UTR—miR-145 was predicted to target two sites in the 3'-UTR of SOX9, located at positions 266–288 and 1386–1408 (Fig. 3A). Reporter constructs containing full-length SOX9 3'-UTR were made in which the sites were deleted individually or in combination. Expression of the 266–288 mutant reporter (site 1 deletion) was 2-fold higher than that of the wild type; however, deletion of the 1386–1408 binding site (site 2) did not affect luciferase activity (Fig. 3B). Simultaneous deletion of both binding sites had no further effect, suggesting that the region 266–288 (site 1) is the one required for the observed miRNA-mediated repression. Furthermore, miR-145 overexpression reduced by 2-fold the expression of a luciferase reporter containing wild type SOX9 3'-UTR. This effect was completely lost by the deletion of the putative 266–288 miR-145 binding site, thus confirming site 1 as the relevant one for the observed miRNA-mediated repression (Fig. 3C).

miR-145 Regulates Expression of Key Cartilage Genes and microRNAs in Human Articular Chondrocytes—To further investigate the functional significance of miR-145, we checked the effect of its overexpression on the production of the key cartilage-specific matrix molecules by human articular chon-

drocytes obtained from six different patients. Levels of direct SOX9 target genes COL2A1 and aggrecan, both essential to normal cartilage function, were significantly reduced by miR-145 overexpression under both normoxic and hypoxic conditions (Fig. 4, A and B). Hypertrophy markers RUNX2 and MMP13 were both decreased by hypoxia but subsequently increased by miR-145 overexpression (Fig. 4, C and D, respectively). We have previously shown that expression of primary miR-675 transcript H19 is dependent on SOX9 and that miR-675 overexpression rescues COL2A1 levels in SOX9-depleted cells, indicating that regulation of COL2A1 by SOX9 is mediated, at least in part, by miR-675 (12). In the present study, overexpression of miR-145 markedly reduced both H19 and miR-675 levels in human chondrocytes, probably indirectly through SOX9 down-regulation (Fig. 5, A and B). However, miR-675 overexpression was not enough to restore COL2A1 levels after miR-145 overexpression (data not shown), indicating that miR-145 may be affecting COL2A1 levels through pathways other than the SOX9 → H19 → miR-675 axis. Interestingly, expression of the other known SOX9-dependent miRNA in cartilage (miR-140) was also reduced by miR-145 overexpression (Fig. 5C), although levels of aggrecanase ADAMTS5, a proposed target of miR-140 were not significantly altered (Fig. 5D). As a form of control, we observed that miR-21-3P, a non-SOX9-dependent miRNA not implicated in cartilage function, was unaffected by manipulation of miR-145 (data not shown).

DISCUSSION

SOX9 is essential for normal cartilage development and function (1, 18). Much is known about the downstream targets of SOX9. In addition to direct activation of all the major tissue-specific matrix genes that give the tissue its function (load-bearing and articulation) (19, 20), recent work has shown that SOX9 also activates transcription of two key microRNAs in human cartilage (miR-140 and miR-675), which mediate important functions in the tissue (11, 12). Due to this extensive regulation of the chondrocyte phenotype by SOX9, identifying upstream regulators of this gene is critical both to our understanding of cartilage homeostasis and for developing new therapeutic approaches to cartilage repair. Although we have previously shown that transcription factor HIF-2 α up-regulates SOX9 in hypoxia (4), relatively little is known about the all-important upstream regulators of this gene. Here we show for the first time in human articular chondrocytes that expression of SOX9 is subject to post-transcriptional regulation by an miRNA (miR-145) binding to a specific site in its 3'-UTR.

In the present study, of the two predicted miR-145 binding sites in the human SOX9 3'-UTR, only one (which is not conserved in mice) was shown to be targeted by miR-145. Deletion of the second putative binding site (which is conserved in mice) from luciferase constructs harboring full-length human SOX9 3'-UTR had no effect on luciferase expression in human chondrocytes. Interestingly, a most recent study reports an effect of miR-145 on Sox9 levels in a murine cell line (C3H10T1/2) undergoing growth factor-induced chondrogenic differentiation (21). Although Yang *et al.* (21) observed altered luciferase expression in response to modulation of miR-145 using con-

miR-145 Directly Targets SOX9 in Human Chondrocytes

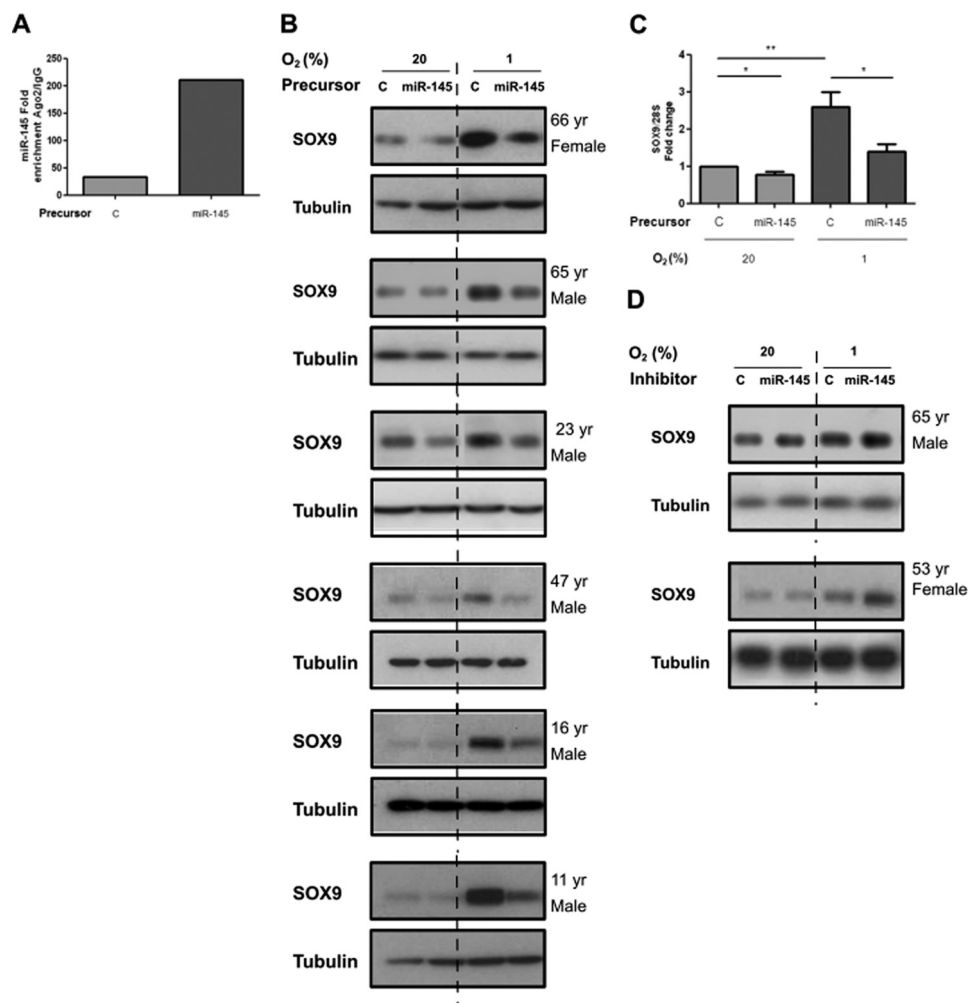


FIGURE 2. miR-145 negatively regulates endogenous SOX9 expression in human articular chondrocytes. *A*, miR-145 overexpression results in a 6-fold increase in the amount of miR-145 bound in the effector RNA-induced silencing complex in human chondrocytes as measured following immunoprecipitation using an anti-Agonate2 antibody and comparing with IgG controls (denoted as C). *B*, Western blots showing reduced SOX9 levels following miR-145 overexpression in first passage chondrocytes from six different donors. Cells were transfected with control (denoted as C) or miR-145 precursor and subsequently grown in 20 or 1% O₂ for 40 h. *C*, SOX9 mRNA levels in response to miR-145 overexpression. Values are presented as relative to that obtained in cells transfected with the control precursor grown in 20% O₂. Data are the average (\pm S.E.) of measurements from nine different experiments (*, $p < 0.05$; **, $p < 0.01$). *D*, Western blots showing increased SOX9 levels following miR-145 inhibition. Cells were transfected with control or miR-145 inhibitor and subsequently grown in 20 or 1% O₂ for 40 h.

structs containing isolated copies of this conserved predicted miR-145 binding site, assays were not performed in the context of full-length Sox9 3'-UTR. Therefore, binding in the mouse system still needs to be assessed under more endogenous conditions, *i.e.* using full-length Sox9 3'-UTR sequences. Nevertheless, if Sox9 proves to be directly targeted by miR-145 in mice (albeit by a different site than in humans), then tissue-specific deletion studies will be most informative to delineate the role of this microRNA in cartilage *in vivo* including investigation of its potential role in osteoarthritis using suitable mouse models.

In humans, the avascular cartilage is hypoxic, and we have previously shown that hypoxia promotes the differentiated articular chondrocyte phenotype through HIF-2 α -mediated induction of SOX9 (4). Interestingly, overexpression of miR-145 appeared to have a greater impact in hypoxic conditions and largely prevented the increase in SOX9 expression induced by hypoxia. Nevertheless, we found that levels of HIF-2 α remain unchanged after miR-145 overexpression (data not

shown), and steady-state levels of miR-145 were not altered in hypoxia. Therefore, it is likely that the hypoxia-induced regulation of SOX9 via HIF-2 α occurs independently of miR-145. On the other hand, miRNA activity may alter without changes in their steady-state levels, *e.g.* by regulation of the accessibility to their target mRNA by RNA-binding proteins in response to different stimuli (22, 23). Whether hypoxia affects the capacity of miR-145 to modulate gene expression remains to be investigated.

The mechanisms maintaining articular chondrocyte homeostasis are perturbed in osteoarthritis, in which chondrocytes recapitulate certain aspects of endochondral ossification including increased expression of transcription factor RUNX2, which promotes hypertrophy, leading to elevated levels of type II collagen-degrading metalloproteinase MMP13 (24, 25). Interestingly, in the present study, these two genes (RUNX2 and MMP13) were both decreased by hypoxia but subsequently increased by miR-145 overexpression. The normal healthy articular chondrocyte phenotype is maintained with a low level

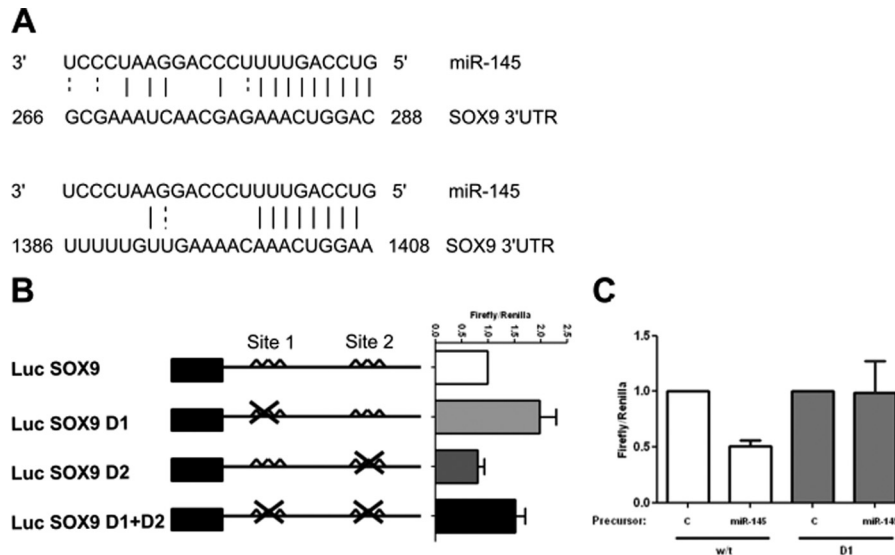


FIGURE 3. miR-145 directly inhibits SOX9 expression in human chondrocytes through a specific binding site located in 3'-UTR. A, schematic representation of the two predicted miR-145 binding sites; numbers indicate the position in the human SOX9 3'-UTR. B, human articular chondrocytes from three different patients were transfected with luciferase (*Luc*) reporters containing full-length SOX9 3'-UTR with or without the indicated deletions: D1, nucleotides 272–293 (containing predicted binding site 1) and D2, nucleotides 1349–1427 (containing predicted binding site 2). Values were plotted relative to that obtained for the wild type (w/t) 3'-UTR. Only deletion of D1 affected luciferase activity. C, deletion of D1 completely abolished the luciferase repression caused by miR-145 overexpression. Note: Values are expressed relative to that obtained for each construct co-transfected with the control miRNA precursor (denoted by C).

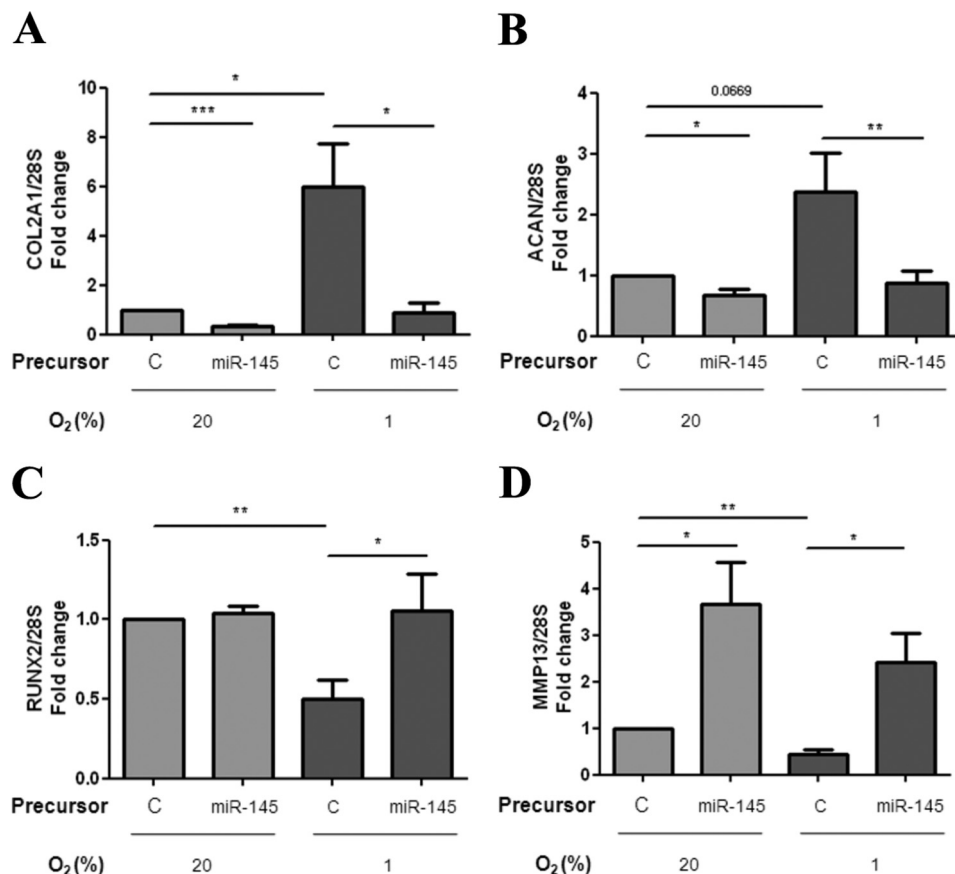


FIGURE 4. miR-145 overexpression significantly alters levels of key SOX9-dependent matrix genes and hypertrophy markers in human articular chondrocytes. Cells were transfected with control (denoted as C) or miR-145 precursor, and RNA was extracted after culture for 40 h in 20 or 1% O₂ tension. Relative levels for each transcript were measured by RT-qPCR. A and B, expression of cartilage-specific and SOX9-dependent matrix genes COL2A1 (A) and aggrecan (ACAN) (B) was significantly decreased by miR-145 overexpression. C and D, in contrast, expression levels of hypertrophy markers RUNX2 (C) and MMP13 (D), which were decreased by hypoxia, were both significantly increased by miR-145 overexpression in hypoxic conditions. Values represent the average (\pm S.E.) of nine different experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

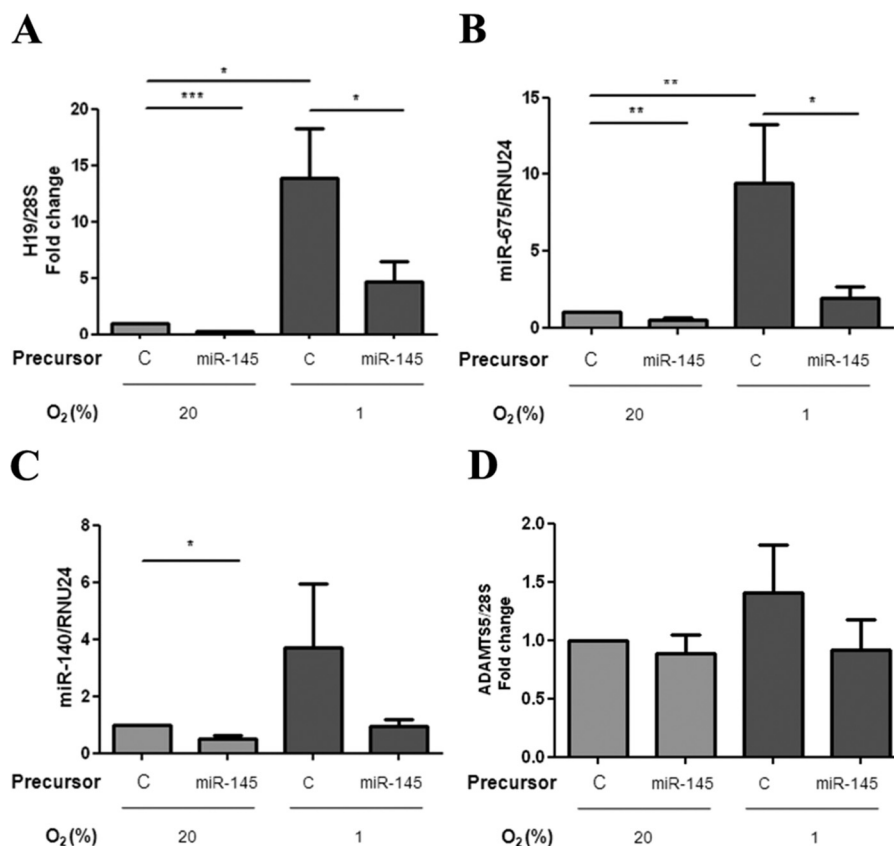


FIGURE 5. miR-145 overexpression significantly reduces expression of key cartilage-specific microRNAs. Human articular chondrocytes were transfected with control (denoted as C) or miR-145 precursor, and RNA was extracted after 40 h of culture in 20 or 1% O₂ tension. Relative levels for each transcript were measured by RT-qPCR. *A* and *B*, expression of both miR-675 primary transcript H19 (*A*) and the mature microRNA transcript (miR-675) (*B*) was significantly decreased by miR-145 overexpression. *C* and *D*, similarly, miR-140 levels were reduced by miR-145 overexpression (*C*); however, ADAMTS5, a putative target of miR-140, was unaffected (*D*). Values represent the average (\pm S.E.) of nine different experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

of RUNX2 and relatively high SOX9 expression (26). Interestingly, Sox9 has been shown to negatively regulate Runx2 via direct activation of transcription factor Bapx2 in proliferating murine chondrocytes (27), and SOX9 expression is known to decrease prior to chondrocyte hypertrophy in the growth plate (28, 29). Furthermore, SOX9 expression levels are reduced in osteoarthritic cartilage when compared with age-matched (non-diseased) controls (29). Hattori *et al.* (30) performed some very informative experiments by overexpressing Sox9 specifically in hypertrophic chondrocytes in mice (using a BAC-Col10a1 promoter) and demonstrated that Sox9 is a negative regulator of endochondral ossification, and hypertrophic markers such as Mmp13 were down-regulated in Sox9-overexpressing hypertrophic chondrocytes. Hence, it is reasonable to speculate that maintenance of SOX9 levels in articular chondrocytes, at least partially mediated by miR-145, is pivotal not just to maintenance of the articular phenotype but to prevention of the hypertrophy-related changes that occur in osteoarthritis. Interestingly, in profiling experiments measuring levels of a total of 723 miRNAs in human articular chondrocytes, miR-145 was one of only three miRNAs found to be significantly up-regulated in cultured osteoarthritic chondrocytes when compared with non-diseased controls (31). The extent to which miR-145 levels are altered in arthritic tissue itself remains to be determined, in particular in the early localized lesions, which may be more informative of initiating events.

We report that miR-145 levels are abundant in human articular chondrocytes where SOX9 is specifically expressed and has an essential role in tissue function and homeostasis. However, miR-145 has been shown to be expressed in various mouse tissues (although cartilage was not tested), being highly abundant in fat and the aorta, and loss of miR-145 induced structural modifications specifically in the proximal aorta, where the aorta emerges from the heart (15). This is intriguing because the aortic valves in the heart express Sox9, which when deleted in the mouse leads to calcification of the aortic valves, the most prominent form of valvular disease (32). Significantly increased expression of Runx2 and other osteogenic markers was detected in these Sox9-deficient aortic valves. Thus, SOX9 appears to play a strikingly similar role in maintaining cellular phenotype and preventing hypertrophy and calcification in two very different tissues, articular cartilage and aortic heart valves, and the possible role of miR-145 in heart valve function and pathology may be an important area for future investigation.

SOX9 has been targeted in studies aimed at treatment of hyaline articular cartilage damage, including Sox9 cDNA transfected in mesenchymal stem cells (33) or viral transduction with SOX9 in passaged osteoarthritic articular chondrocytes (34). Through upstream miRNA targeting, here we offer a way of manipulating SOX9 expression without resorting to gene therapy and its associated hazards. Furthermore, the relative ease by which miRNAs can be manipulated pharmacologically

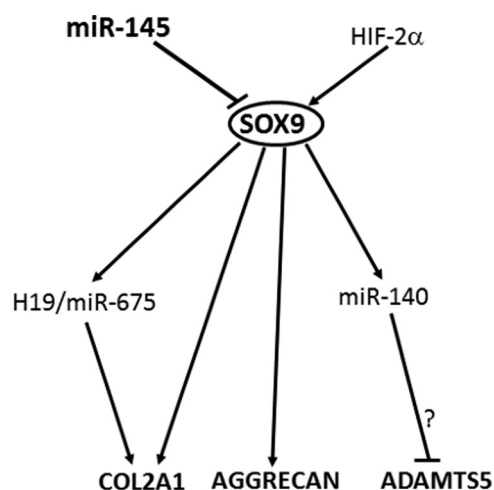


FIGURE 6. Transcriptional and microRNA-mediated regulation of SOX9 and cartilage matrix gene expression in human articular chondrocytes. miR-145 directly targets (inhibits) essential master regulator SOX9, which exerts profound phenotypic effects by up-regulation of two key cartilage-specific microRNAs in addition to directly targeting the essential matrix genes.

provides distinct therapeutic opportunities (35). There are several tools available to selectively target miRNA pathways *in vivo* including both synthetic mimics and inhibitors. Anti-miR inhibitors are modified antisense oligonucleotides with full or partial complementarity of a mature miRNA that can reduce the endogenous levels of an miRNA. Currently there are over 70 clinical trials worldwide (completed or actively recruiting) based on miRNA manipulation to treat a range of conditions including various cancers and cardiovascular diseases; however, there are none to date for arthritis (source: www.clinicaltrials.gov). MicroRNAs normally simultaneously regulate the expression of several targets, and experiments to determine other direct targets of miR-145 are currently being carried out in our laboratory. From a therapeutic viewpoint (with regard to both efficacy and safety), it will be important to identify all such directly targeted pathways before proceeding with clinical trials.

In summary, we show that cartilage master regulator SOX9 is subject to significant post-transcriptional regulation by a specific microRNA (miR-145), which is highly expressed in human articular chondrocytes. This novel microRNA-mediated regulation involves a network involving complex interactions between microRNAs and key transcription factors and exerts profound phenotypic effects in human chondrocytes (Fig. 6). The extent to which these phenotypic changes are a direct consequence of miR-145 targeting of SOX9 remains to be determined. Importantly, our data suggest that manipulation of SOX9-targeting miR-145 in chondrocytes could provide a much needed successful treatment for cartilage injuries and potentially for the chronic disease of osteoarthritis, a condition for which there is still no disease-modifying treatment.

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REFERENCES

- Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrughe, B. (1999) Sox9 is required for cartilage formation. *Nat. Genet.* **22**, 85–89
- Foster, J. W., Dominguez-Steglich, M. A., Guioli, S., Kwok, C., Weller, P. A., Stevanovi, M., Weissenbach, J., Mansour, S., Young, I. D., and Goodfellow, P. N. (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* **372**, 525–530
- Murphy, C. L., and Polak, J. M. (2004) Control of human articular chondrocyte differentiation by reduced oxygen tension. *J. Cell. Physiol.* **199**, 451–459
- Lafont, J. E., Talma, S., and Murphy, C. L. (2007) Hypoxia-inducible factor 2α is essential for hypoxic induction of the human articular chondrocyte phenotype. *Arthritis Rheum.* **56**, 3297–3306
- Haag, J., Gebhard, P. M., and Aigner, T. (2008) SOX gene expression in human osteoarthritic cartilage. *Pathobiology* **75**, 195–199
- Lefebvre, V., Huang, W., Harley, V. R., Goodfellow, P. N., and de Crombrughe, B. (1997) SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro α1(II) collagen gene. *Mol. Cell. Biol.* **17**, 2336–2346
- Sekiya, I., Tsuji, K., Koopman, P., Watanabe, H., Yamada, Y., Shinomiya, K., Nifuji, A., and Noda, M. (2000) SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6. *J. Biol. Chem.* **275**, 10738–10744
- Alvarez-Garcia, I., and Miska, E. A. (2005) MicroRNA functions in animal development and human disease. *Development* **132**, 4653–4662
- Jones, S. W., Watkins, G., Le Good, N., Roberts, S., Murphy, C. L., Brockbank, S. M., Needham, M. R., Read, S. J., and Newham, P. (2009) The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-α and MMP13. *Osteoarthritis Cartilage* **17**, 464–472
- Miyaki, S., Nakasa, T., Otsuki, S., Grogan, S. P., Higashiyama, R., Inoue, A., Kato, Y., Sato, T., Lotz, M. K., and Asahara, H. (2009) MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. *Arthritis Rheum.* **60**, 2723–2730
- Miyaki, S., Sato, T., Inoue, A., Otsuki, S., Ito, Y., Yokoyama, S., Kato, Y., Takemoto, F., Nakasa, T., Yamashita, S., Takada, S., Lotz, M. K., Ueno-Kudo, H., and Asahara, H. (2010) MicroRNA-140 plays dual roles in both cartilage development and homeostasis. *Genes Dev.* **24**, 1173–1185
- Dudek, K. A., Lafont, J. E., Martinez-Sanchez, A., and Murphy, C. L. (2010) Type II collagen expression is regulated by tissue-specific miR-675 in human articular chondrocytes. *J. Biol. Chem.* **285**, 24381–24387
- Nakamura, Y., Yamamoto, K., He, X., Otsuki, B., Kim, Y., Murao, H., Soeda, T., Tsumaki, N., Deng, J. M., Zhang, Z., Behringer, R. R., Crombrughe, B., Postlethwait, J. H., Warman, M. L., Nakamura, T., and Akiyama, H. (2011) Wwp2 is essential for palatogenesis mediated by the interaction between Sox9 and mediator subunit 25. *Nat. Commun.* **2**, 251
- Xin, M., Small, E. M., Sutherland, L. B., Qi, X., McAnally, J., Plato, C. F., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2009) MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. *Genes Dev.* **23**, 2166–2178
- Elia, L., Quintavalle, M., Zhang, J., Contu, R., Cossu, L., Latronico, M. V., Peterson, K. L., Indolfi, C., Catalucci, D., Chen, J., Courtneidge, S. A., and Condorelli, G. (2009) The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. *Cell Death Differ.* **16**, 1590–1598
- Xu, N., Papagiannakopoulos, T., Pan, G., Thomson, J. A., and Kosik, K. S. (2009) MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. *Cell* **137**, 647–658
- Chekulaeva, M., and Filipowicz, W. (2009) Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr. Opin. Cell Biol.* **21**, 452–460
- Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A., and de Crombrughe, B. (2002) The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* **16**, 2813–2828
- Bell, D. M., Leung, K. K., Wheatley, S. C., Ng, L. J., Zhou, S., Ling, K. W., Sham, M. H., Koopman, P., Tam, P. P., and Cheah, K. S. (1997) SOX9 directly regulates the type-II collagen gene. *Nat. Genet.* **16**, 174–178

20. Han, Y., and Lefebvre, V. (2008) L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. *Mol. Cell. Biol.* **28**, 4999–5013
21. Yang, B., Guo, H., Zhang, Y., Chen, L., Ying, D., and Dong, S. (2011) MicroRNA-145 regulates chondrogenic differentiation of mesenchymal stem cells by targeting Sox9. *PLoS One* **6**, e21679
22. Kedde, M., van Kouwenhove, M., Zwart, W., Oude Vrielink, J. A., Elkon, R., and Agami, R. (2010) A Pumilio-induced RNA structure switch in p27–3' UTR controls miR-221 and miR-222 accessibility. *Nat. Cell Biol.* **12**, 1014–1020
23. Kedde, M., Strasser, M. J., Boldajipour, B., Oude Vrielink, J. A., Slanchev, K., le Sage, C., Nagel, R., Voorhoeve, P. M., van Duijse, J., Ørom, U. A., Lund, A. H., Perrakis, A., Raz, E., and Agami, R. (2007) RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell* **131**, 1273–1286
24. Billingham, R. C., Dahlberg, L., Ionescu, M., Reiner, A., Bourne, R., Rora-beck, C., Mitchell, P., Hambor, J., Diekmann, O., Tschesche, H., Chen, J., Van Wart, H., and Poole, A. R. (1997) Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J. Clin. Invest.* **99**, 1534–1545
25. Wang, X., Manner, P. A., Horner, A., Shum, L., Tuan, R. S., and Nuckolls, G. H. (2004) Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. *Osteoarthritis Cartilage* **12**, 963–973
26. Yang, K. G., Saris, D. B., Geuze, R. E., van Rijen, M. H., van der Helm, Y. J., Verbout, A. J., Creemers, L. B., and Dhert, W. J. (2006) Altered *in vitro* chondrogenic properties of chondrocytes harvested from unaffected cartilage in osteoarthritic joints. *Osteoarthritis Cartilage* **14**, 561–570
27. Yamashita, S., Andoh, M., Ueno-Kudoh, H., Sato, T., Miyaki, S., and Asahara, H. (2009) Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. *Exp. Cell Res.* **315**, 2231–2240
28. Zhao, Q., Eberspaecher, H., Lefebvre, V., and De Crombrughe, B. (1997) Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Dev. Dyn.* **209**, 377–386
29. Tew, S. R., Clegg, P. D., Brew, C. J., Redmond, C. M., and Hardingham, T. E. (2007) SOX9 transduction of a human chondrocytic cell line identifies novel genes regulated in primary human chondrocytes and in osteoarthritis. *Arthritis Res. Ther.* **9**, R107
30. Hattori, T., Müller, C., Gebhard, S., Bauer, E., Pausch, F., Schlund, B., Bösl, M. R., Hess, A., Surmann-Schmitt, C., von der Mark, H., de Crombrughe, B., and von der Mark, K. (2010) SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. *Development* **137**, 901–911
31. Diaz-Prado, S., Cicione, S., Muinos, C., Arufe, E., Carmen Fuentes, Ma., de Toro, I., and Blanco, F. J. (2009) in *Arthritis & Rheumatism, Volume 60, October 2009 Abstract Supplement*, pp. 742, American College of Rheumatology, Atlanta, GA
32. Peacock, J. D., Levay, A. K., Gillaspie, D. B., Tao, G., and Lincoln, J. (2010) Reduced sox9 function promotes heart valve calcification phenotypes *in vivo*. *Circ. Res.* **106**, 712–719
33. Tsuchiya, H., Kitoh, H., Sugiura, F., and Ishiguro, N. (2003) Chondrogenesis enhanced by overexpression of sox9 gene in mouse bone marrow-derived mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* **301**, 338–343
34. Cucchiari, M., Thurn, T., Weimer, A., Kohn, D., Terwilliger, E. F., and Madry, H. (2007) Restoration of the extracellular matrix in human osteoarthritic articular cartilage by overexpression of the transcription factor SOX9. *Arthritis Rheum.* **56**, 158–167
35. van Rooij, E., Sutherland, L. B., Qi, X., Richardson, J. A., Hill, J., and Olson, E. N. (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316**, 575–579